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# Identical mutations in three different fibroblast growth factor receptor genes in autosomal dominant craniosynostosis syndromes

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Pfeiffer syndrome (PS; McKusick MIM 101600) is an autosomal dominant craniosynostosis syndrome with characteristic craniofacial anomalies and broad thumbs and big toes<sup>1,2</sup>. We have previously demonstrated genetic heterogeneity in PS and mapped a gene to chromosome 8 (ref. 3) and a second to chromosome 10 (ref. 4). The gene on chromosome 8 is the fibroblast growth factor receptor 1 (*FGFR1*) with a common mutation (C755G) predicting a Pro252Arg substitution<sup>5</sup>. The gene on chromosome 10 is *FGFR2* with several different mutations causing sporadic and familial PS<sup>4,6-8</sup> (Table 1). We report a recurrent single point mutation in the *FGFR3* gene, located on chromosome 4p, in ten unrelated families with craniosynostosis syndromes. This mutation (C749G) predicts a Pro250Arg amino acid substitution in the extracellular domain of the *FGFR3* protein. Interestingly, this common mutation occurs precisely at the analogous position within the *FGFR3* protein as the mutations in *FGFR1* (Pro252Arg) and *FGFR2* (Pro253Arg) previously reported in Pfeiffer<sup>5</sup> and Apert<sup>9</sup> syndromes, respectively.

To identify additional genes involved in craniosynostosis syndromes, we performed linkage studies in two families (Fig. 1a,b) where some of the clinical findings were most consistent with PS. These studies excluded both chromosomes 8 and 10. Analysis of these families was consistent with linkage to 4p16.3, in the same region as *FGFR3* (data not shown). We used *FGFR3* exon specific PCR primer pairs to amplify genomic DNA from affected individuals from these families and the PCR products were screened for mutations by heteroduplex analysis. Analysis of a 341-bp PCR amplification product designed to include exon 7 of *FGFR3* revealed heteroduplex alterations in both families (data not shown). DNA sequencing of this PCR product from one affected individual revealed a C to G transversion at position 749 of the coding cDNA sequence, located between the second and third putative immunoglobulin-like (Ig) domains of the *FGFR3* protein (Fig. 2a). The C to G change predicts a Pro250Arg substitution. This mutation also creates a new *NciI* restriction site within the 341-bp PCR fragment

allowing rapid screening of DNA from all available family members. The disease phenotype cosegregated with the mutant allele in both families (Fig. 2b). In contrast, the mutation was not detected in over 120 normal chromosomes. We next screened DNA from 65 unrelated individuals with craniosynostosis with or without limb involvement and were able to identify an additional eight samples with this mutation. On clinical examination, five families segregated non-syndromic craniosynostosis, the remaining three had some clinical findings that were consistent with PS, Crouzon, or Saethre-Chotzen syndrome. The disease phenotype cosegregated with the *FGFR3* mutation in all families in which additional family members' DNA samples were available for testing (6 of 8; data not shown). Thus, we found a total of ten independent cases with the Pro250Arg mutation in *FGFR3*. The phenotypic spectrum of individuals with this mutation varied from bilateral coronal craniosynostosis (Fig. 1a-c) to unilateral coronal craniosynostosis (plagiocephaly) to macrocephaly. This variation could be seen even within the same family. Most affected individuals had normal appearing hands and feet on clinical exam. However, on X-ray, the most consistent findings were short and broad middle phalanges of the fingers and absent or hypoplastic middle phalanges of the toes (Fig. 1d,e). Carpal and tarsal fusion was also noted. We propose that this unique mutation in *FGFR3* is quite frequent, especially among patients whose bilateral or unilateral coronal craniosynostosis does not fit into any of the classic craniosynostosis syndromes.

Similarly, a wide clinical spectrum has recently been described in two large kindreds with non-syndromic craniosynostosis which were linked to 4p<sup>10,11</sup>. This may suggest the possibility of the same *FGFR3* mutation in these two families. Since previously characterized mutations in *FGFR3* are known to be involved in skeletal dysplasias with short stature<sup>12-15</sup> our affected individuals carrying the Pro250Arg mutation in *FGFR3* were examined for height and limb length; all were within the normal range. Thus, non-syndromic craniosynostosis and Crouzon syndrome with acanthosis nigricans<sup>16</sup> are examples of disorders without problems in long bone development that are caused by mutations in *FGFR3*.

These observations establish that identical mutations in three different *FGFR* genes can cause autosomal dominant craniosynostosis syndromes. It is remarkable that mutations in *FGFR1* causing PS and in *FGFR3* causing non-syndromic craniosynostosis both result in nucleotide and amino acid substitutions which are analogous to the Pro253Arg mutation in *FGFR2* found in Apert syndrome<sup>9</sup> (Fig. 3). The observation of identical mutations in three separate *FGFR* genes points to a common pathogenesis. It will be interesting to address: i) how mutations in three separate *FGFR* genes result in similar phenotypes; ii) the effect of this specific arginine for proline substitution on *FGFR* function; and iii) how mutations of adjacent amino acids in *FGFR3* (Fig. 3) result in such distinct phenotypes.

**Table 1** *FGFR* mutations in Pfeiffer syndrome and non-syndromic craniosynostosis

	Refs
<i>FGFR1</i>	
Pro252Arg (11)	4,5,8
<i>FGFR2</i>	
Cys278Phe <sup>a</sup> (2)	8
Asp321Ala <sup>a</sup> (1)	6
Thr341Pro (1)	7
Cys342Arg <sup>b</sup> (11)	4,7,8
Cys342Ser (1)	8
Cys342Tyr <sup>a</sup> (1)	7
Ala344Pro (1)	8
Val359Phe (1)	8
Acceptor splice site, exon 9 (5)	4,6
Intron (5' donor, exon 9) <sup>c</sup> (1)	8
<i>FGFR3</i>	
Pro250Arg (10)	This study

Numbers in parentheses indicate the number of independent cases reported. <sup>a</sup>Also found in Crouzon syndrome. <sup>b</sup>Also found in Crouzon and Jackson-Weiss syndrome. <sup>c</sup>6-bp insertion results in a 51-bp deletion of exon 9 due to activation of a cryptic donor splice site. A different mutation with an identical splicing error has also been detected in Crouzon syndrome<sup>25</sup>.

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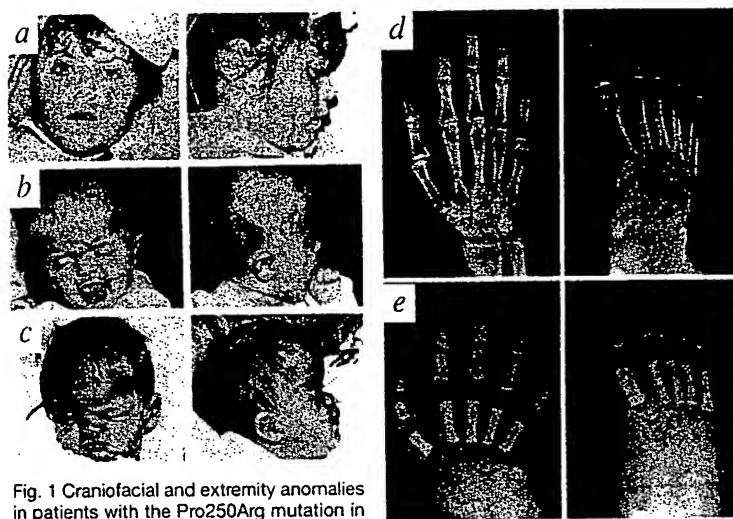


Fig. 1 Craniofacial and extremity anomalies in patients with the Pro250Arg mutation in *FGFR3*. a, Proband (IV.2) in kindred PS 12 (Fig. 2b) with bilateral coronal synostosis. Her brother and mother also have craniosynostosis. b, Proband (III.1) as an infant in family PS 2 (Fig. 2b) with bilateral coronal synostosis. She has short broad middle phalanges of her fingers, coned epiphyses of proximal phalanges of toes 2, 3, and 4, and tarsal fusion. Her father has craniosynostosis with normal X-rays of hands, small middle phalanges of the second toes and absent middle phalanges of the fifth toes. c, Proband with bilateral coronal synostosis. A sibling also has bilateral coronal synostosis. Their father has macrocephaly without evidence of craniosynostosis. d and e, On X-ray all three have short broad middle phalanges of fingers and father and proband have absent middle phalanges of the toes.

Recent studies have demonstrated that an *FGFR2* mutation (Cys342Tyr) found in Pfeiffer and Crouzon syndromes<sup>17</sup> and *FGFR3* mutations in achondroplasia<sup>18,19</sup> and type 1 (TD-1) and type 2 (TD-2) thanatophoric dysplasia<sup>19</sup> result in ligand-independent, constitutive activation of the receptor. Furthermore, the phenotype of *Fgfr3* knockout mice includes overgrowth of the long bones and axial skeleton suggesting that

*FGFR3* is a negative regulator of bone growth<sup>20,21</sup>. However, individuals with non-syndromic craniosynostosis caused by *FGFR3* Pro250Arg mutations have normal long bone growth, suggesting a distinct pathogenesis.

There are several possible explanations for the different phenotypic effects of the *FGFR3* mutations found in the chondrodysplasias and those of the *FGFR3* Pro250Arg mutation in non-syndromic craniosynostosis. First, the Pro250Arg mutation may alter ligand binding so as to induce receptor activation by an inappropriate ligand whose expression is tissue specific (such as growth plate versus cranial suture). Alternatively, the Pro250Arg mutation may cause ligand-independent activation of downstream intracellular *FGFR3* signalling pathways (activation of phospholipase C  $\gamma$ , Ras-mediated activation of MAP kinases, and internalization and nuclear transport of receptor bound FGF<sup>22,23</sup>). However, different *FGFR3* mutations may have differential effects on the intensity and/or specificity of signal transduction through each of these pathways and the developmental importance of each pathway may vary in different tissues. Finally, the Pro250Arg mutation may enhance the ability of *FGFR2* to form heterodimers with other *FGFRs* resulting in either ligand-dependent or independent receptor activation. *FGFR1*, *FGFR2*, and *FGFR3* are expressed in different temporospatial patterns in the developing mouse but share some areas of overlap which include the germinal epithelium of the neural tube and prebone cartilage rudiments of all the bones<sup>24</sup>. It is possible that the *FGFR1* Pro252Arg and *FGFR3* Pro250Arg mutations influence *FGFR2* signalling, thereby causing the craniosynostosis phenotype. The observation that patients with *FGFR1* or *FGFR3* mutations tend to have a milder phenotype than those seen with *FGFR2* mutations<sup>4</sup> and that Apert syndrome,

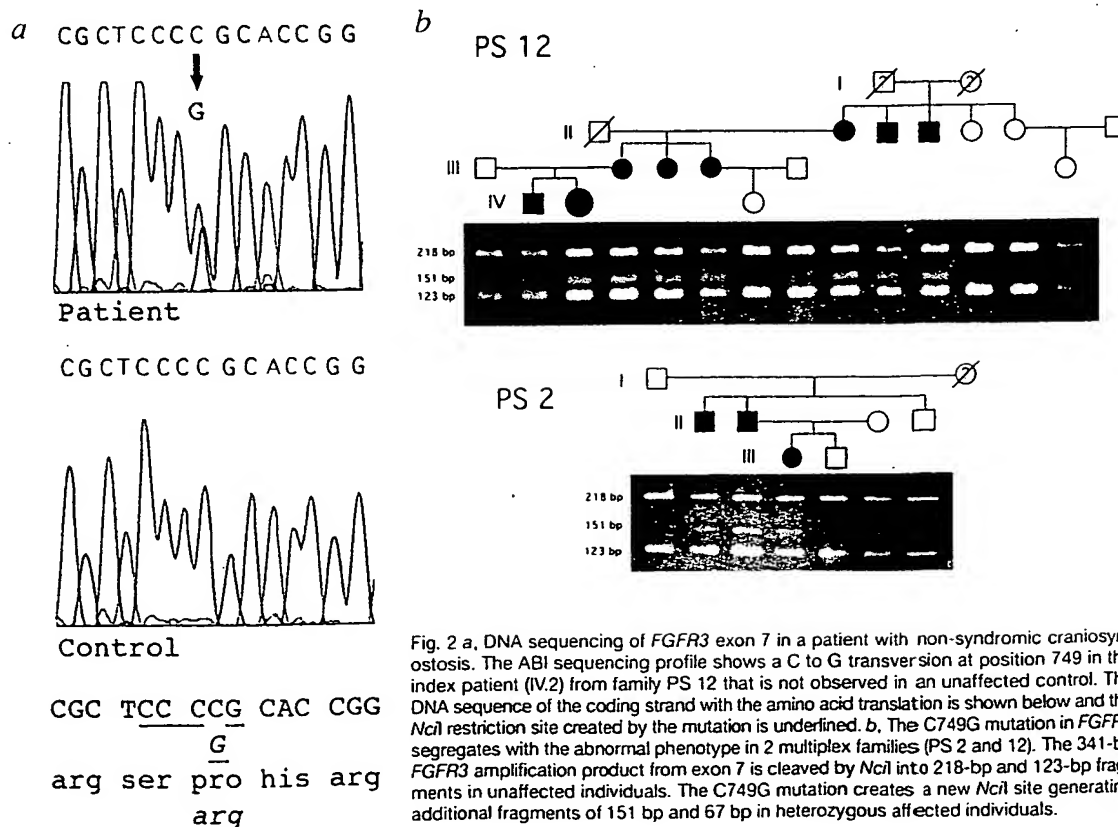


Fig. 2 a, DNA sequencing of *FGFR3* exon 7 in a patient with non-syndromic craniosynostosis. The ABI sequencing profile shows a C to G transversion at position 749 in the index patient (IV.2) from family PS 12 that is not observed in an unaffected control. The DNA sequence of the coding strand with the amino acid translation is shown below and the *NciI* restriction site created by the mutation is underlined. b, The C749G mutation in *FGFR3* segregates with the abnormal phenotype in 2 multiplex families (PS 2 and 12). The 341-bp *FGFR3* amplification product from exon 7 is cleaved by *NciI* into 218-bp and 123-bp fragments in unaffected individuals. The C749G mutation creates a new *NciI* site generating additional fragments of 151 bp and 67 bp in heterozygous affected individuals.

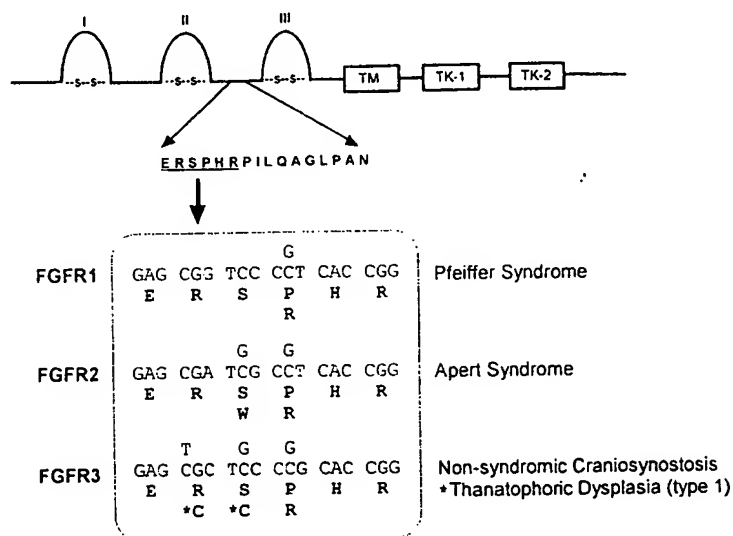


Fig. 3 Schematic diagram of the general organization of a fibroblast growth factor receptor with three extracellular Ig-like domains (I-III), transmembrane domain (TM) and split intracellular tyrosine kinase domain (TK-1 & TK-2). The conserved sequence of 16 amino acids (identical in all four mammalian FGFRs) in the linker region between the second and third Ig-like domains is shown. The nucleotide sequence of the first 6 codons of this conserved amino acid sequence in FGFR1, 2 and 3 is illustrated below. The nucleotide and amino acid substitutions found in Pfeiffer<sup>5</sup>, Apert syndrome<sup>9</sup>, non-syndromic craniosynostosis (this report) and thanatophoric dysplasia type 1 (refs 14,26,27) are indicated.

the most severe phenotype in the spectrum of *FGFR2* mutations, is caused by the equivalent Pro252Arg mutation in *FGFR2* is consistent with this possibility. Cotransfection studies using *FGFR* mutant constructs and transgenic mouse models will allow testing of these hypotheses and further elucidate the role of fibroblast growth factor receptors in normal bone development.

## Methods

**Patients.** Two unrelated families (kindreds 2 and 12, Fig. 2b) with some clinical findings consistent with PS have been described<sup>3</sup>. An additional 65 patients were evaluated through the genetics clinic at the Children's Hospital of Philadelphia and referred by

other medical geneticists. The majority were referred as having sporadic PS (32 of 65) or familial PS (8 of 65). The remainder had some findings consistent with Saethre-Chotzen (3), Crouzon syndrome (2), or non-syndromic craniosynostosis (20 of 65). Only those DNA samples which were negative for mutations in *FGFR1* and *FGFR2* (or not yet tested) were included.

**DNA analysis.** Genomic DNA samples were analysed by PCR amplification. Preliminary linkage studies were performed in families PS 2 and PS 12 using PCR primer pairs from the chromosomal region for *FGFR1* on 8p (D8S278, D8S255, GATA8G08, D8S285; ref. 3), *FGFR2* on 10q (D10S221, D10S190, D10S209, D10S587; ref. 4), and *FGFR3* on 4p (D4S412, GATA2G12, D4S1627, Research Genetics, Inc.). PCR primers were designed to amplify *FGFR3* from genomic DNA as described<sup>15</sup>. The forward and reverse primers used to amplify exon 7 (191 bp) and 150 bp of flanking intron sequence are 5'-CGGCAGTGACG-GTGGTGGTGA-3' and 5'-CCAAATCCTCAGCAACCC-3', respectively. PCR reactions were performed using Perkin Elmer reagents and the recommended standard conditions except that DMSO was added to a final concentration of 10%. Reactions were carried out in a Hybaid Omnigene Temperature Cycler. The program consisted of an initial denaturing step (94 °C, 5 min) followed by 35 PCR cycles (94 °C, 30 s; 60 °C, 45 s; 72 °C, 45 s) and a terminal extension step (72 °C, 10 min). Heteroduplex analysis was carried out as described previously<sup>15</sup>. PCR products were purified using the Wizard spin column kit (Promega) and sequenced using the fluorescent dideoxy terminator method of cycle sequencing on a PE/ABD 373a automated DNA sequencer following ABI protocols. Restriction digests were carried out with *NciI* (New England Biolabs) according to the manufacturer's recommendations. Digestion products were analyzed by gel electrophoresis on 2% Nusieve/1% agarose gels and visualized by staining with ethidium bromide.

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